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Immunoaffinity Purification of Dietary Heterocyclic Amine Carcinogens

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Cooking meats produces a family of heterocylic aromatic amines that are carcinogens in rodents and genotoxic in many short-term assays. Concern that these compounds may be human carcinogens has prompted us to develop immunochemical methods for quantifying these compounds in the human diet and for identifying the parent compounds and metabolites in urine and feces. Previously reported monoclonal antibodies to 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 6-phenyl-2-amino-1-methylimidazo[4,5-*f*]pyridine (PhIP) were used to purify by immunoaffinity these known mutagens and cross-reacting structural analogs from well-done cooked beef and urine samples. Materials recovered from the immunoaffinity columns were subsequently separated by HPLC to purify the known mutagens from cross-reacting chemicals that co-purify by immunoaffinity. Immunoaffinity chromatography was found to be a rapid means of quantifying individual known mutagens, with a minimum of precolumn sample clean-up required. In addition, this procedure has yielded several new mutagens present in cooked meats that are apparently structural analogs of PhIP. Immunoaffinity techniques were also used to purify metabolites from the urine of rats and humans exposed to MeIQx or PhIP. For MeIQx-exposed rats, the combination antibodies immunoconcentrated 75% of the total urinary radioactivity. Analysis of PhIP metabolites recovered from antibody columns is facilitated by the intrinsic fluorescence of PhIP and its metabolites, providing sufficient sensitivity to monitor individuals for the levels of PhIP excreted following consumption of typical western diets.

Introduction

Cooking meats produces a family of heterocylic aromatic amines that are carcinogens in rodents and positive in many short-term genotoxicity assays (*1,2*). Among these, 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) accounts for most of the bacterial mutagenicity in beef, and 6-phenyl-2-amino-1-methylimidazo[4,5-*f*]pyridine (PhIP) is the most abundant in beef. We have previously reported on the development of monoclonal antibodies with varying selectivities toward the heterocyclic amines 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), MeIQx, and PhIP (*3–5*). A method for detecting urinary metabolites of these compounds using immunoaffinity chromatography followed by HPLC has also been reported (*6*). Advantage can be taken of PhIP's intrinsic fluorescence; many of its metabolites are also fluorescent (Vanderlaan et al., submitted), which provides a very sensitive detection method for this heterocyclic compound.

It is unlikely that DNA or blood protein adducts will be suitable for monitoring human exposure to heterocyclic amines because they are formed at levels that are too low for detection by currently available methods. As demonstrated in this paper, however, the detection of human urinary metabolites is possible. Urinary metabolites are evidence of recent exposures (previous 24–48 hr) and are ideal for intervention studies, where both the diet and excreta can be measured for the same compounds. As such, the methods reported here should find application in determining the factors influencing human uptake and metabolism of heterocyclic amine carcinogens.

Materials and Methods

Monoclonal antibodies AIA-1, AIA-2, PhIP-1, and PhIP-4 were immobilized and used to immunochemically purify chemicals as described previously (*6*). Materials could be recovered from all the antibody columns in 1:1 MeOH:H_2O except for AIA-2. AIA-2 was refractile to the recovery of bound chemicals unless 100% methanol was used. Known mutagens were separated from cross-reacting chemicals that co-immunoaffinity purify by HPLC, and the same HPLC conditions were used for all of the results presented here: 0.01 M triethylamonium acetate buffer (pH 7.2) with a methanol gradient (5–90% over 80 min) on a PRP-1 column. UV absorption was monitored at 315 nm for PhIP and 275 nm for MeIQx. Fluorescence was monitored at 325 nm excitation and 375 nm emission. Fractions were collected at 1-min intervals, dried, and tested on bacterial mutagenesis assays using strain TA98 and microsomal activation.

In an effort to increase the yield of these PhIP-like compounds formed in beef by frying, raw beef was spiked with 4% creatinine and with 4% phenylalanine before cooking. These compounds have been shown to be precursors for PhIP, and it was expected

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that addition of these compounds would increase the yield of PhIP and perhaps increase the yield of structurally related analogs of PhIP that could be purified with PhIP-4.

Results and Discussion

PhIP

By far the most important result to date is that PhIP is only one of a family of closely related structural analogs formed by cooking in fried beef. Figure 1 shows the analysis of materials recovered from well-done cooked beef using immunoaffinity purification with PhIP-1 or PhIP-4. Figure 1*A* shows the UV absorbance trace of material retained by PhIP-1, and Figure 1*B* shows a similar chromatogram for PhIP-4-retained materials. PhIP elutes with a retention time near 69 min in both chromatograms and is the major peak recovered from PhIP-1. In contrast, PhIP-4 immunopurifies at least a dozen compounds, and PhIP is not the principal material. Shown in Figure 1*B* are the results of bacterial mutagenicity assays. There are multiple mutagens present, and PhIP is only a minor mutagen in this family of compounds. Peaks in the mutagenicity do not match the retention time of other known food mutagens, and PhIP-4 does not retain standards of the known mutagens other than PhIP, suggesting that these compounds are not among those currently known to occur in well-done cooked beef. Separation of PhIP-4 immunoaffinity-purified material from creatinine and phenylalanine spiked, well-done beef is shown in Figure 1*C*. The relative amount of PhIP recovered from the immunoaffinity column was increased, but the other compounds were not increased by this method, suggesting that other precursors are involved in their formation.

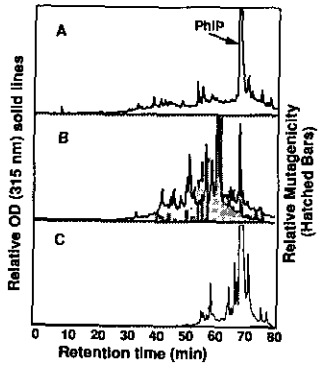

FIGURE 1. HPLC chromatograms of immunoaffinity-purified 6-phenyl-2-amino-1-methylimidazo[4,5-*f*]pyridine (PhIP)-related compounds: (*A*) 315 nm absorption trace of PhIP-1 retained compounds from well-done fried beef; (*B*) 315 nm absorption trace of PhIP-4 retained compounds and Salmonella mutagenicity of fractions (shaded bars) from well-done fried beef; (*C*) 315 nm absorption trace of PhIP-4 retained compounds from beef spiked with creatinine and phenylalanine before being fried well-done.

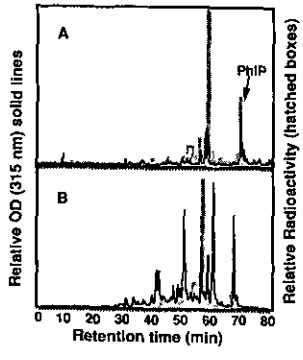

FIGURE 2. HPLC chromatograms of immunoaffinity-purified PhIP-related compounds from urine of rats exposed to radiolabeled PhIP (*A*) and to cooked-beef extract spiked with radiolabeled PhIP (*B*).

Urine from rats exposed to PhIP and beef extract with PhIP showed materials that could be purified with PhIP-1 and PhIP-4 antibodies. In this case, the fluorescence properties of PhIP allow detection of PhIP metabolites at levels comparable to those of human exposures. Figure 2*A* shows the results obtained for PhIP-4 affinity column purification of urine from rats exposed to radiolabeled PhIP alone. In addition to recovering unmetabolized PhIP, six more polar metabolites elute with retention times of 50–60 min. All the fluorescent compounds are radioactive and vice versa. It should be noted that these metabolites are not the major metabolites of PhIP, which elute earlier and are not retained by the affinity column (Vanderlaan et al., submitted).

The results shown in Figure 2*B* were obtained for the PhIP-4 analysis of rat urine when the radiolabeled PhIP was given in beef extract. The radioactive metabolites eluting with retention times of 50–60 min are similar relative to PhIP (Fig. 2*A*), showing that the presence of the beef extract did not dramatically influence the formation of these metabolites. This observation suggests that the multitude of compounds present in the beef extract do not modify PhIP metabolism. In addition, there are now several peaks, two with retention times near 43 min, one at 52 min, and one at 62 min, which are not radioactive. These presumedly are derived from the cross-reacting PhIP analogs present in the cooked beef, illustrated in Figure 1*B*. Thus, the PhIP analogs in cooked beef can be in absorbed from the gut and excreted in the urine.

MeIQx

Analysis of cooked-beef extracts with either AIA-1 or AIA-2 immunopurifies only MeIQx and 4,8-DiMeIQx. Limited efforts

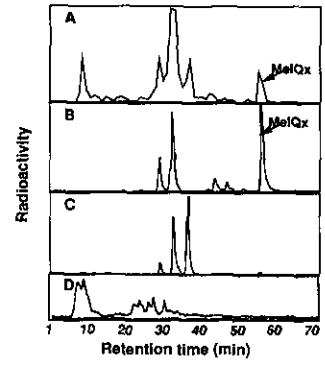

FIGURE 3. HPLC chromatograms of immunoaffinity-purified ^{14}C-MeIQx and its metabolites from the urine of exposed rats. (*A*) Whole rat urine concentrated on C-18 only; (*B*) compounds retained by monoclonal antibody AIA-1; (*C*) additional compounds retained by monoclonal antibody AIA-2 following depletion of the urine by AIA-1; (*D*) compounds not retained by either antibody.

to isolate additional structural analogs of MeIQx from commercial beef broths have also been negative. In contrats to PhIP, MeIQx appears not to be one of a larger family of compounds. The finding that AIA- 1 did not purify more compounds from well-done beef is somewhat surprising because previous characterization of this antibody in competition ELISA has shown that it cross-reacted with many meat mutagens (*3*). AIA-2, in contrast, is highly specific for MeIQx in ELISA.

Rat urinary metabolites of MeIQx are, however, readily recovered from AIA-1 and AIA-2 affinity columns. Figure 3 shows the results obtained from urine of rats exposed to ^{14}C-MeIQx. Figure 3*A* shows the analysis of total urinary metabolites retained on the C-18 column, without use of any antibody column. Greater than 95% of the total urinary radioactivity is recoverable from the C-18 column. As noted previously (*6*), AIA-1 retains not only MeIQx but also at least four earlier eluting metabolites (Fig. 3*B*). Flow through sample, depleted of AIA-1 binding metabolites, was then applied to the AIA-2 column, and the results are shown in Figure 3*C*. Only a trace amount of MeIQx was recovered by this second column, indicating the efficiency of the first antibody column. In addition, three more metabolites were recovered. The UV absorption traces matched the radioactivity traces exactly for both antibodies, indicating that no nonradioactive materials cross-reacted with the antibodies. Finally, the run through of both columns was chromatographed, with the results shown in Figure 3*D*. About 25% of the starting radioactivity remained, which was made up of only the most polar MeIQx metabolites. The above results show that a combination of the two antibody columns is an effective means of concentrating and purifying MeIQx metabolites from urine.

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